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LETTERS AND CORRESPONDENCE

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Rapid Detection of Glucose-6-Phosphate Dehydrogenase Type A-$^{202A/376G}$ Deficiency by Allele-Specific Polymerase Chain Reaction

To the Editor: Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked trait, affects more than 400 million people worldwide [1,2]. One of the type A$^-$ G6PD variants occurs with a 10–11% frequency in African-American males and contains two point mutations at nucleotides 202 and 376 (G6PD A-$^{202A/376G}$) [1,2]. Rapid screening for the defect is required in a variety of clinical conditions [3].

We have developed a rapid, inexpensive and reliable method for the detection of the G6PD A-$^{202A/376G}$ variant. The technique uses allele-specific polymerase chain reaction (AS-PCR), first described for the diagnosis of sickle cell anemia [4]. To differentiate between normal and deficient variants, the 3′-terminal nucleotide of the sense primers were synthesized to either match or mismatch the point mutations at positions 202 and 376. A match or mismatch between the 3′-terminal nucleotide of the primer and the template DNA determines the amplification efficiency of the PCR. The following primers were used in the assay: 202G (5′-CCGAAAACACCTTCATC**G**-3′) hybridizes to normal DNA; 202A (5′-CCGAAAACACCTTCATC**A**-3′) is complementary to the variant G6PD A-$^{202A/376G}$ DNA; 5′-ACCTGTGGGTCCTGGTC-3′ is the antisense oligonucleotide for both the 202G and 202A sense primers; 376A (5′-CTCAACAGCCACATG**A**-3′) hybridizes to normal DNA; 376G (5′-CTCAACAGCCACATG**G**-3′) is complementary to the variant G6PD A-$^{202A/376G}$ DNA; 5′-GAGAAGAGGACCTTTGCTTTAC-3′ is the antisense primer for both the 376A and 376G sense primers. We also synthesized a set of control primers, sense primer C (5′-CACCTTCAAGGAGCCCTTTG-3′) and antisense primer 5′-GCAAGACTCACTTCCTGATCCC-3′, which amplify part of G6PD exon 7.

Two different strategies were developed to screen for G6PD A-$^{202A/376G}$ (Fig. 1). The first tests DNA for the 202 and 376 mutations individually and requires five reactions (one reaction for each primer set) to identify the deficient genotype. Figure 1A and B illustrate the amplification pattern for each primer pair when reacted with either normal or G6PD A-$^{202A/376G}$ DNA. A second, simpler method (Fig. 1C) tests only for the mutation at nucleotide 202 and requires only two reactions. This is sufficient for screening because the nucleotide 202 mutation does not occur without the

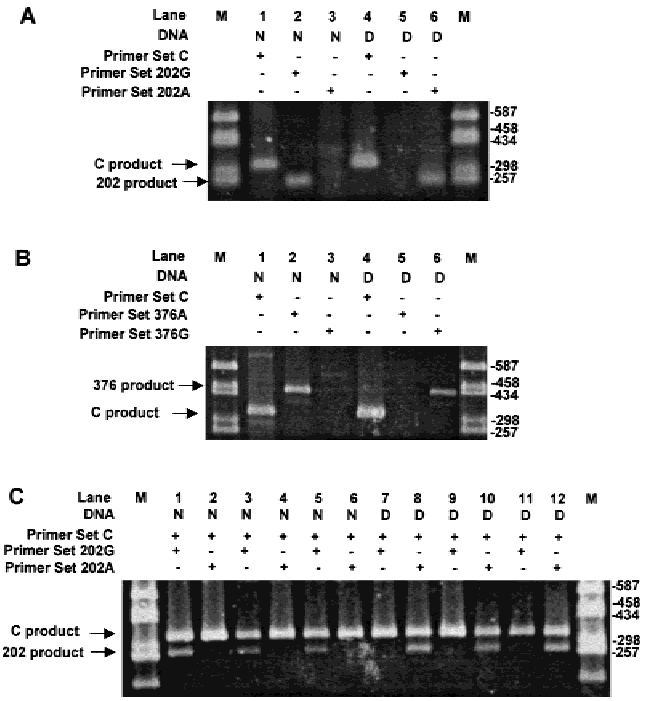

Fig. 1. Identification of G6PD A-$^{202A/376G}$ with AS-PCR. DNA from normal (N) or deficient (D) patients were isolated and amplified using allele-specific primers. The PCR mixture contained the following components: 150–300 ng genomic DNA, 0.5 μM each primer (0.2 μM of control primers in Gel C reactions), 200 μM each dNTP, 10 μL of 10× PCR buffer, 2.5 units Taq polymerase, 1 mM $MgCl_2$ (1.5 mM for Gel C reactions), in 0.1 mL final volume (Gibco BRL, Grand Island, NY). Samples were subjected to 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 50°C for 30 sec, and 72°C for 30 sec, with a final extension step at 72°C for 7 min. Gel A shows the allele-specificity of the 202G (lanes 2 and 5) and 202A (lanes 3 and 6) sense primers. Gel B shows the allele-specificity of the 376A (lanes 2 and 5) and 376G (lanes 3 and 6) sense primers. Gel C shows the rapid detection of G6PD A-$^{202A/376G}$ deficiency using an internal positive control in DNA samples from three normal and three deficient patients. All lanes show the amplification of DNA by the control primers ("C product"). Presence or absence of a second PCR product ("202 product") result from either a match or mismatch between the allele-specific 202 primer set and the DNA template. Lanes marked M represent DNA base pair (bp) markers. Arrows depict the PCR products using control primers, "C product" (333-bp), and allele-specific primers, "202 product" (266-bp) and "376 product" (452-bp).

simultaneous presence of the second mutation at nucleotide 376 [2,5]. In this assay, the PCR mixture contained the 202 allele-specific primers and the control primers together. The use of the control primers served as an indicator of the efficiency of the PCR amplification in individual tubes.

Our assay was successful in screening 14 patients for G6PD A-$^{202A/376G}$. No false positives or negatives were generated when compared to the results obtained by the method of Beutler et al., which uses PCR amplification followed by restriction endonuclease digestion [2]. The presented technique has an advantage over the endonuclease digestion method because it requires only PCR followed by gel electrophoresis. It can also be used to identify heterozygous females with type A-$^{202A/376G}$ G6PD deficiency, which is not feasible using the endonuclease digestion technique. Our method may also be useful for rapid identification of the defect after blood loss or hemolysis when screening by the determination of RBC G6PD activity is not reliable due to the relatively high enzyme activity in deficient RBC newly released from the bone marrow.

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Severe Hemolytic Anemia Following High-Dose Intravenous Immunoglobulin Administration in a Patient With Kawasaki Disease

To the Editor: We describe a patient with Kawasaki disease (KD) who developed severe acute autoimmune hemolytic anemia (AIHA) following high-dose intravenous immunoglobulin (IVIG) administrations.

A 5-month-old Japanese female infant, weighing 6,020 gm, was referred for treatment of KD. The patient's family history was unremarkable. Laboratory examination on admission revealed leukocytosis (18,600/μL), hypoalbuminemia (28 g/L, or 2.8 g/dL) and a high C-reactive protein (CRP) (0.23 g/L, or 23.7 mg/dL) level. There was no evidence of anemia. On the day of admission (5 days after the onset of symptoms), we started intravenous administration of 400 mg/kg/day of intact immunoglobulin for 5 days and 5 mg/kg/day of flurbiprofen. However, neither clinical symptoms nor laboratory data improved after therapy. We administered another 2 g/kg of immunoglobulin intravenously 12 days after the onset. In spite of the additional IVIG administration, two-dimensional echocardiogram (2-

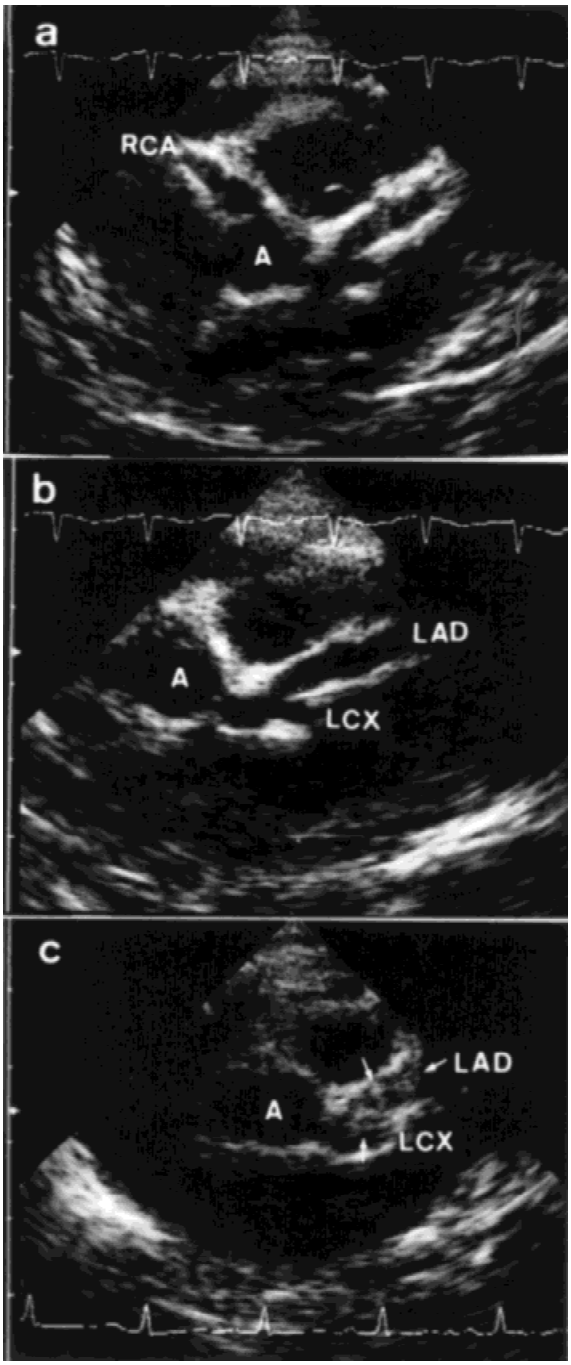

Fig. 1. Two-dimensional echocardiographs (2-DE) performed on the 15th day (a,b) and 29th day (c) following the onset of symptoms. The 2-DE on the 15th day demonstrated large coronary aneurysms in the bilateral coronary arteries, but without evidence of thrombus. However, several thrombi (arrows in c) were detected in the left coronary artery on the 29th day. A, aorta; LAD, left anterior descending artery; LCX, left circumflex artery; R, right coronary artery.

DE) performed on the 15th day detected large coronary aneurysms (Fig. 1a,b). The patient was observed to have anemia (red blood cell (RBC), 2.55 × $10^6/\mu L$; hemoglobin, 68 g/L, or 6.8 g/dL) on the 16th day. Further examination revealed the presence of anti-A antibodies (1: 512), and positive direct and indirect Coombs tests. The patient's blood type was A(+), and we were unable to perform blood transfusions. On the 21st day, the RBC and hemoglobin concentrations decreased to 1.89 × $10^6/\mu L$ and 48 g/L (or 4.8 g/dL), respectively, prompting us to start intravenous administration of prednisone (1.5 mg/kg/day). Following treatment with steroids, clinical symptoms and laboratory values, including RBC, hemoglobin, white blood cell count, and CRP, improved. However, on the 29th day, the 2-DE showed the development of thrombi in left coronary aneurysm (Fig. 1c). We immediately began anticoagulant therapy, including dipryridamole (4 mg/kg), heparin, and urokinase (10,000 U/kg). However, she suffered an acute myocardial infarction involving the anterolateral wall and apex on the 33rd day. Intracoronary administration of recombinant alteplase was performed, resulting in an increase in the fractional shortening from 0.16 to 0.32. The patient was subsequently treated with warfarin potassium, dipyridamole, and flurbiprofen. However, the direct Coombs test remained positive.

The AIHA in this patient may have been caused by anti-A antibodies based on positive direct and indirect Coombs tests. According to previous reports, the AIHA caused by high-dose IVIG administration for idiopathic thrombocytopenic purpura or KD can be mediated by cold agglutinating anti-RBC antibodies [1,2], immunodisorders associated with KD [3], or attachment of antibodies to RBC membranes [4,5], and, therefore, is usually self-limited. However, the AIHA in this patient was so serious that it was necessary to give steroid hormones, which may have developed thrombi in the coronary aneurysms. The AIHA probably developed because the patient received a total 4 g/kg dose of immunoglobulin. Because the AIHA caused by anti-A or anti-B antibodies is dose dependent, the incidence and severity of AIHA may increase with higher total doses of immunoglobulin. The clinical course of this patient emphasizes the fact that retreatment with high-dose IVIG can cause severe AIHA.

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Philadelphia Chromosome Positive Acute Lymphocytic Leukemia Arising From Aplastic Anemia

To the Editor: We report a case of Philadelphia chromosome (Ph^1) positive acute lymphocytic leukemia (ALL) arising from aplastic anemia (AA).

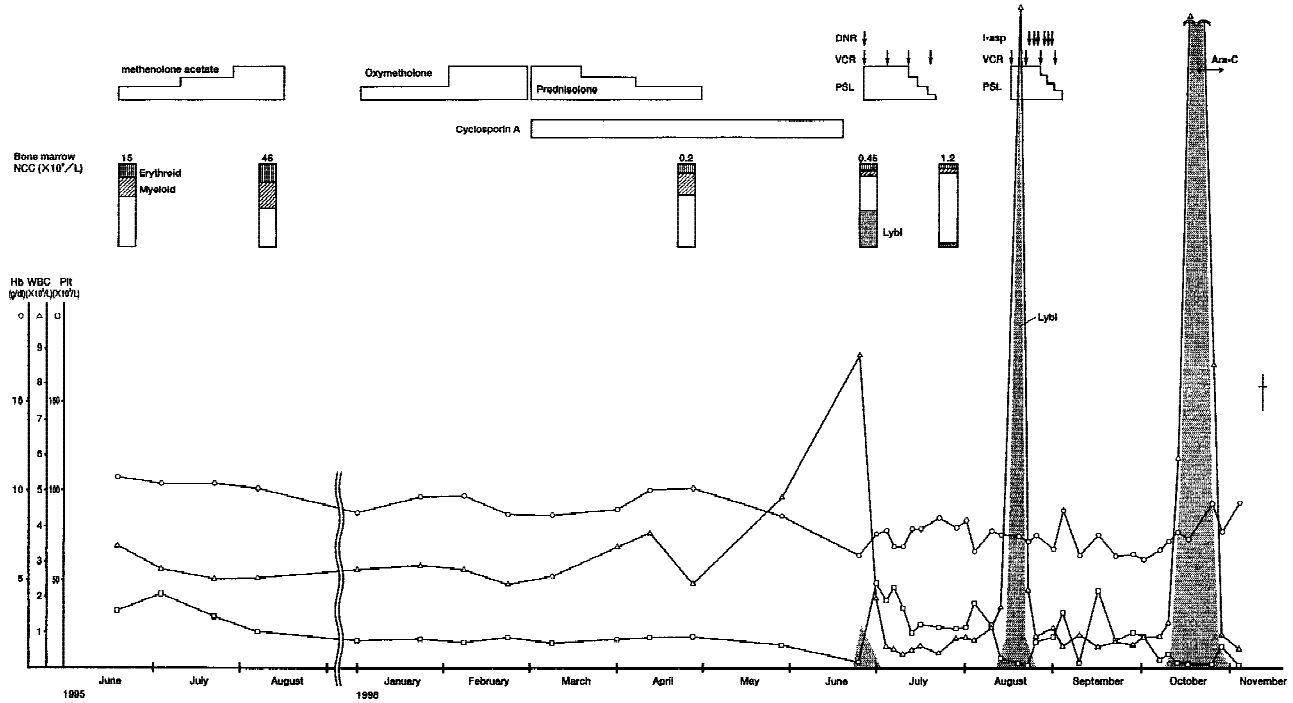

Fig. 1. Clinical course. Cy A, cyclosporin A; DNR, daunorubicin; VCR, vincristine; PSL, prednisolone; 1-asp, 1-asparaginase; Ara-C, cytosine arabinoside; Lybl, lymphoblast.

Pancytopenia was detected in a 54-year-old woman at her regular medical check-up in June 1995. The hematologic values were as follows: hemoglobin (Hb), 10.8 g/dL; white blood cell count (WBC), 3.6×10^9/L (neutrophils 33%); and platelet count (PLT), 35×10^9/L. A bone marrow examination revealed marked hypocellularity with no increase in the number of immature precursors or any evidence of dysplastic changes. Chromosomal analysis of the bone marrow cells showed 46,XX in all 20 bone marrow cells examined. A diagnosis of AA was made. She was treated with methenolone acetate for 2 months, yielding no hematologic response. She discontinued the medication because of adverse effects. In January 1998, she was treated with oxymetholone, and subsequently, cyclosporin A and prednisolone. However, no response was observed. In June 1998, 36 months from the initial diagnosis, she developed a systemic bleeding tendency. Her hematologic values were as follows: Hb, 6.1 g/dL; WBC, 8.8×10^9/L (neutrophils 10%, lymphocytes 80%, lymphoblasts 7%, monocytes 3%); and Plt, 2×10^9/L. Examination of bone marrow revealed hypoplastic marrow with 38.8% lymphoblasts. The blasts expressed pre-B-cell markers (CD10, CD19, CD20, HLA-DR, TdT positive), without any myeloid or T-cell lineage markers. Chromosomal analysis revealed 46,XX,t(9;22)(q34;q11) in 13 of 20 cells examined. Reverse transcription polymerase chain reaction (RT-PCR) analysis proved the presence of the minor bcr-abl fusion mRNA, while the major bcr-abl fusion was not detected. A diagnosis of Ph^1 positive hypoplastic ALL was made. She was treated with chemotherapy. However, she did not achieve a remission and died from sepsis in December 1998. The clinical course is outlined in Figure 1.

Several studies suggest that AA patients have molecular evidence of clonal hematopoiesis [1,2]. In addition a high incidence of myelodysplastic syndrome (MDS) or acute myelocytic leukemia (AML) arising from AA and a chromosomal abnormality, monosomy 7, was reported in patients with AA treated with immunosuppressive therapy and G-CSF [3]. Although the long-term use of these agents is thought to be a risk factor for the development of MDS or AML, it is uncertain whether the clonal origin of these diseases is present from the onset of AA or is induced during treatment. On the other hand, it was reported previously that major bcr–abl fusion transcripts are detectable in the blood of 30% of healthy individuals [4] and that minor bcr–abl fusion transcripts are also detectable in two thirds of healthy adults [5]. The significance of these findings is unknown, and it is not clear whether these transcripts are from continuously arising de novo translocations or due to the presence of a small clone. Although we could not confirm it in our case, the leukemic clone carrying the minor bcr–abl translocation may have been present far earlier than the onset of ALL and proliferated during immunosuppressive therapy.

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